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ORIGINAL RESEARCH ARTICLE

Stimulation of secondary metabolism in grape berry exocarps by a nature-based strategy of foliar application of polyols

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ABSTRACT

In grapes under drought stress, polyols accumulate through tight coordination at the molecular level between increased membrane transport of polyols and inhibition of polyol oxidation. Here, the effects on grape metabolism of an exogenous foliar application of polyols as a potential sustainable viticultural practice to increase grapevine performance and berry quality were thoroughly assessed. Grapevines were pulverised with a polyol solution containing 2 mM mannitol and 2 mM sorbitol, and the metabolome of grape berry exocarps and important metabolic pathways associated with berry quality and polyol metabolism were analysed at *véraison* and mature stages. By combining metabolomics analysis using UPLC-MS, enzyme activity assays and targeted transcriptional analyses, it was demonstrated that foliar application of polyols stimulated by 3.5-fold and 6-fold abscisic acid (ABA) biosynthesis at *véraison* and mature grape berries, respectively. It also stimulated the concentration of anthocyanins, stilbenes and total phenolics in exocarps through the upregulation of phenylpropanoid, stilbenoid and anthocyanin biosynthetic pathways shown by increases in phenylalanine-ammonia lyase (PAL) activity (3-fold) and *VviPAL1* expression, stilbene synthase 1 (*VviSTS1*) transcripts (ca. 5-fold), UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) activity and *VviUFGT1* expression, among other results, at the mature stage, when these changes were most noticeable. Many secondary metabolites synthesised in these pathways identified by UPLC-MS analysis were present at higher quantities in exocarps from polyol-treated plants such as fertaric acid, *E*-resveratrol, *E*-piceatannol, piceid, pallidol, *E*-ε-viniferin, myricetin-hexoside 1, cyanidin-3-*O*-glucoside and malvidin-3-O-(6-*p*-coumaroyl)-glucoside. Foliar application of low-concentration polyols is, therefore, a promising biostimulant-based strategy to improve grape berry quality and nutritional value in the current context of climate change.

KEYWORDS: Grape berry quality, polyols, biostimulants, secondary metabolism, sustainable viticultural practices

INTRODUCTION

Vitis vinifera L. is one of the most valuable fruit species worldwide, representing an important role in the socio-economic life of wine-producing regions. However, in the global climate change scenario, viticulture faces new challenges and threats, considering that the productivity and the quality of grapes, and consequently of wine, depends on several climate factors. The most typical abiotic stresses observed include water deficit (drought), salinity, high temperature and excessive radiation (Bernardo *et al.*, 2018). In this context, the development of efficient strategies to mitigate stresses and/or diseases while improving berry quality has been a relevant topic of research in the grapevine (Bernardo *et al.*, 2018; Monteiro *et al.*, 2022; Samuels *et al.*, 2022).

Exogenous compounds that act as protectants, hormones, nutrients, biostimulants and elicitors have been successfully used to improve grapevine tolerance against environmental stress (Monteiro *et al.*, 2022). This is the case in the foliar application of kaolin, a chemically inert mineral with excellent reflective properties that reduces the impact of heat and drought in vineyards while improving berry quality because of many molecular and biochemical changes in key secondary metabolic pathways (Conde *et al.*, 2016). A plant biostimulant is any substance (or microorganism) applied to plants to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content (Bodin *et al.*, 2020). Bioactive molecules called elicitors, such as methyl jasmonate, ABA, glycine betaine and salicylic acid, are also biostimulants that regulate primary and secondary plant metabolism (Monteiro *et al.*, 2022). Moreover, osmoprotectant compounds such as amino acids, sugars and polyols can also be considered biostimulants of plants.

In particular, polyols, also known as sugar alcohols, play a crucial and multifaceted role in plants facing various environmental stresses, particularly water stress and salinity. These organic compounds act as essential components of the plant's defence mechanisms, enabling it to adapt and thrive in adverse conditions. In water-stressed vines and other stressed plants, polyols function as vital osmoprotectants (Conde *et al*., 2015). When subjected to water deficit or high salinity levels, plants experience reduced water availability and increased osmotic stress (Yancey *et al*., 1982). Under such circumstances, polyols step in to maintain cellular turgor and regulate osmotic potential. By acting as compatible solutes, they ensure that cells retain sufficient water, preventing cellular dehydration and sustaining critical cellular functions (Ashraf and Harris, 2004). In the context of salinity stress, polyols aid in ion homeostasis. Excess salt in the soil disrupts cellular ion balance and can have detrimental effects on cellular metabolism (Munns and Tester, 2008). Polyols play a crucial role in osmotic adjustment, reducing the impact of salt on cellular water potential and preventing water loss from cells. Additionally, they facilitate the compartmentalisation of toxic ions, such as sodium and chloride, within vacuoles, preventing their harmful effects on cellular functions. This capacity to regulate ion homeostasis contributes significantly to the stress tolerance of water-stressed vines and other plants under saline conditions. Furthermore, polyols showcase their remarkable antioxidant properties in stressed plants. When plants face stress, such as water deficit or salinity, metabolic imbalances can lead to the accumulation of reactive oxygen species (ROS). These ROS can inflict oxidative damage on cellular components, including lipids, proteins and DNA, leading to reduced plant productivity and growth (Gill and Tuteja, 2010). Polyols serve as effective antioxidants, scavenging ROS and mitigating oxidative stress. By minimising the harmful effects of ROS, polyols protect plant cells and enable them to maintain viability and functionality under stress.

The accumulation of osmoprotectant solutes, such as polyols (e.g., mannitol, sorbitol, myo-inositol, galactinol and dulcitol), has also been demonstrated as an important biochemical and physiological adaptation mechanism that is triggered when grapevine senses water deficit conditions (Conde *et al.*, 2015). In particular, mannitol and sorbitol intracellular accumulation in response to drought occurs by the coordination of mannitol and sorbitol dehydrogenase activities with VviPLT1 (VvPMT5)-mediated polyol transport, which results in the inhibition of mannitol and sorbitol oxidation into fructose and stimulation of the reverse reaction (Conde *et al.*, 2015). However, while the role of polyols in response to osmotic imbalances in grapevine is well described, their potential use as exogenously applied biostimulants able to increase grapevine plasticity/adaptability to its surrounding environmental conditions and/or to improve quality-related traits, for instance, by stimulating secondary metabolism in berries, is so far completely unknown.

Secondary metabolites are extremely important to the quality traits of wine, contributing to its colour, flavour, texture and aroma but also in plant adaptation to environmental changes. The synthesis of phenolic compounds occurs via the general phenylpropanoid pathway and subsequent conversion into stilbenes, flavonoids or phenolic acids (Rienth *et al.*, 2021). PAL, chalcone synthase (CHS), stilbene synthase (STS), anthocyanin reductase (ANR), leucoanthocyanin reductase (LAR) and UFGT are all branch point enzymes involved in the biosynthesis of grape polyphenols under the regulation of MYB transcription factors (Bogs *et al.*, 2005; Czemmel *et al.*, 2012). Besides being regulated by ABA during ripening, the synthesis of secondary metabolites resulting from these complex pathways is known to be dependent on available carbon (C) skeletons derived from primary metabolism and the availability of monosaccharides, such as glucose and fructose (Garrido *et al.*, 2021).

Based on the known beneficial properties of polyols in grapevine, in this work, we hypothesised that the foliar application of a polyol aqueous solution containing 2 mM mannitol and 2 mM sorbitol could improve quality-related traits through the stimulation of the secondary metabolism while enhancing plant plasticity to changes in the surrounding edaphoclimatic conditions. An array of metabolomics,

enzyme activities and transcriptional analysis approaches were used to investigate this hypothesis.

MATERIAL AND METHODS

1. Grapevine Field Conditions, Treatment and Sampling

Grape berry samples were harvested from field-grown "Touriga Nacional" cultivar grapevines (*Vitis vinifera* L.) at 12 years old and 10–12 buds per vine were pruned; grapevines were grafted onto 1103P rootstock, conducted in a double Guyot system, from a 21 ha vineyard at "Escola Profissional do Rodo", in the Baixo Corgo Sub-Region from the Douro Demarcated Region (Denomination of Origin Douro/Porto), located at Peso da Régua, Portugal (41°10'35.80''N; 7°47'48.00''W). The canopy height and thickness were 1.1–1.2 m and 0.5–0.6 m, respectively. The plants were managed without irrigation and grown using standard cultural decisions as applied by commercial farmers.

The climate is typically Mediterranean, with mild rainy winters and long, hot and dry summers (Jones and Alves, 2012). Total annual rainfall is 1112 mm, of which 14.1 % is from June to September. The warmest months are July and August, and the coldest are January and December, with mean daily temperatures of 20.7 °C and 6.1 °C, respectively (data for the period 1991–2021, according to the database of Climate-Data.org).

Mature leaves of 27 plants distributed over three vineyard rows from each of three 0.3 ha plots randomly located throughout the vineyard were manually sprayed (carefully, so the berry clusters were not sprayed) with an aqueous solution containing 2 mM sorbitol and 2 mM mannitol (with 0.05 % Tween-20 as surfactant to improve adhesion). This concentration was chosen because it is not sufficient to promote polyol-induced osmotic stress in leaves and is in the same order of magnitude as the K_m of the polyol transporter VvPLT1/VvPMT5 (also present in leaves) for mannitol (K_m of 5.4) and sorbitol (K_m of 9.5), respectively (Conde *et al*., 2015). The first application was performed in July 2019, at the late green berry phase and right before *véraison* (EL-33). A second application was performed during the *véraison* (EL-35) stage (Coombe, 1995). In each of the three other plots of 0.3 ha, the leaves of 27 plants from three vineyard rows were exogenously treated with an aqueous solution of just 0.05 % Tween-20 serving as the control condition. All grapevine rows were side-by-side (guaranteeing similar edaphoclimatic conditions) on a hill with an N–S orientation.

Leaf gas exchange was measured with an infrared gas analyser (LC pro+, ADC Bioscientific Ltd., UK) operating in the open mode. Measurements were carried out in the morning (9:30–11:30). The photosynthetic photon flux density (PPFD) incident on the leaves was always higher than 1500 μmol m^{-2} s^{-1}, which is above photosynthesis saturation in the considered species (Flexas *et al.*, 2002). Net photosynthetic rate (A), stomatal conductance (g_s), transpiration rate (E) and intercellular CO_2 concentration/ambient CO_2 ratio (C_i/C_a) were calculated using the equations of von Caemmerer and Farquhar (1981). The A/g_s ratio was used as a proxy for intrinsic water use efficiency, according to Iacono *et al.* (1998).

Grape berry sampling was performed at two different stages: *véraison*—EL-35 (after the second application) and mature stage—EL-38. Phenological parameters, such as average berry weight, pH, water content and total soluble sugars (°Brix) were assessed. When sampling *véraison* berries, a representative mix of pigmented and non-pigmented berries was obtained. This careful procedure (as in Conde *et al.* (2016)) was followed both in the cluster and for different clusters of the plant, with half-pigmented and half-non-pigmented berries collected from each condition. Berry ripening rate was similar between conditions with no apparent phenological displacement. No difference in *véraison* pigmented:non-pigmented berries ratio between treatments was evident. For each ripening phase and each experimental condition, three sets of berries (each ranging from 81 to 108 berries in total), one for each of the three experimental plots and considering all precautions described above, were obtained. Each pool (from each plot) represented a biological replicate. Approximately half of each set of berries was ground to a fine powder under liquid nitrogen refrigeration and stored at –80 °C. The berry skin of the remaining grape berries was gently separated from the mesocarp and ground to a fine powder using a mortar and a pestle under liquid nitrogen and stored at –80 °C. When obtaining *véraison* exocarps, a representative mix of pigmented and non-pigmented berries was obtained from each set, with half of pigmented and half of non-pigmented berries used for exocarp separation.

2. ABA quantification

Ground berry tissues of whole berry and exocarps were thoroughly mixed with 500 μL of methanol 100 % in an approximately 1:1 powder:methanol ratio and incubated with constant agitation at 4 °C overnight. Samples were centrifuged for 20 min at 18,000 × *g*, and the supernatants were collected. The supernatants were left in a vacuum drier at 4 °C for 18 h, and the obtained pellet was resuspended in 500 μL Tris-buffered saline 1×. The quantification of ABA was assayed with the Phytodetek Immunoassay Kit for ABA (Agdia Inc., Elkhart, IN, USA) according to the manufacturer's protocol.

3. Quantification of sorbitol and secondary metabolites by high-performance liquid chromatography (HPLC)

The extraction and quantification of primary metabolites were performed as described by Silva *et al.* (2017) and Noronha *et al.* (2021). The liquid extraction of primary solutes was carried out using 100 mg of lyophilised berries samples with 1 mL of ultrapure H_2O and 1 mL of chloroform. This mixture was vortexed for 5 minutes and incubated at 50 °C for 30 minutes with continuous agitation. After incubation, the samples were centrifuged at 15,000 × *g* for 15 min at 4 °C, and the aqueous supernatant (upper layer) was collected. The supernatant was filtered using a PTFE 0.2 μm filter and quantified by HPLC-RI using a Rezex RCM-Monosaccharide

Ca^{2+} (8 %) column (Phenomenex, California, United States of America) using a flow rate of 0.2 mL min^{-1} at 40 °C and water as the mobile phase. The sorbitol concentration of each sample was determined by comparison of the peak area and retention time with standard curves. Mannitol concentration was also tentatively determined but was not detected.

For absolute quantification and characterisation of piceid and cyanidin-3-glucoside, methanolic extracts of grape berries and berries skins were prepared with freeze-dried samples, using a proportion of 1.5 mL of 80 % (v/v) methanol per 25 mg of sample. The methanolic extracts were filtered using 0.22 µm CLARIFY-PTFE 13 mm Syringe filters and prepared for quantification. The samples were separated using a flow rate of 0.8 mL m^{-1} using water:formic acid (99:1) and methanol as mobile phase using an RP18 (25·0.4 mm, particle size of 5 µm, Merck, Darmstadt, Germany) column connected to an HPLC (Hitachi, Ibaraki, Japan) coupled with a diode array detector (DAD). The concentrations of piceid and cyanidin-3-glucoside in each sample were determined by comparison of the area and retention time with standard curves obtained using 5 points/standard solutions derived from > 95 % pure Piceid (Sigma-Aldrich, Missouri, United States of America) and > 95 % pure Cyanidin-3-glucoside (Sigma-Aldrich, Missouri, United States of America) standards, respectively. For piceid quantification, a wavelength of 320 nm was used, whereas 520 nm was used for cyanidin-3-glucoside quantification.

4. Metabolomics analysis of secondary metabolome by Ultra-Performance Liquid Chromatography coupled to Mass Spectrometry (UPLC-MS)

Metabolites of processed grape berries and exocarps were extracted from freeze-dried samples using a ratio of 1.5 mL of 80 % (v/v) methanol per 25 mg of dry weight. Samples were macerated and sonicated for 30 min and left at 4 °C in the dark overnight. The samples were centrifuged at 18,000 × *g* for 10 min, and the supernatants were collected. Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS)-targeted metabolomic analysis was performed as optimised previously (Billet *et al.*, 2018; Martins *et al.*, 2021), using an ACQUITY UPLC system combined with a photodiode array detector and a Xevo TQD mass spectrometer (Waters, Milford, MA, United States) equipped with an electrospray ionisation source regulated by Masslynx 4.2 software. Analyte separation was achieved by using a Waters Acquity HSS T3 C18 column (150 × 2.1 mm, 1.8 µm) with a flow rate of 0.4 mL/min at 55 °C. The injection volume was 5 µL. The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in acetonitrile). Chromatographic separation was carried out using an 18-min linear gradient from 5 to 50 % (v/v) solvent B. MS detection was performed in both positive and negative modes. The capillary voltage was 3000 V and sample cone voltages were 30 and 50 V. The cone and desolvation gas flow rates were 60 and 800 L h^{-1}. Identification of analytes was based on retention times, m/z values and UV spectra in comparison with commercial standards. UPLC–MS analyses were achieved using the selected ion monitoring (SIM) mode of the targeted molecular ions. UPLC-MS analyses were carried out using a selected ion monitoring (SIM) mode of the targeted molecular ions. SIM chromatograms were mathematically integrated using the subprogram QuanLynx 4.2. Peak integration was made using the ApexTrack algorithm with a mass window of 0.1 Da and a relative retention time window of 1 min, followed by Savitzky–Golay smoothing (iteration = 1 and width = 1). To evaluate the robustness of measurements and analytical variability, a pool of samples was prepared from all experimental conditions and maturation phases to obtain a quality control sample (QC) and the samples were randomly injected independently from the treatment or developmental phase. Three QC samples were injected to equilibrate the system before each analytical sample set, and one QC sample was injected every eight samples to check for potential analytical drift. Relative quantification was determined for L-proline (m1), L-iso-leucine (m2), L-leucine (m3), L-phenylalanine (m4), L-tyrosine (m5), L-tryptophane (m6), cyanidin-3-*O*-glucoside (m7), peonidin-3-*O*-glucoside (m8), delphinidin-3-glucoside (m9), petunidin-3-*O*-glucoside (m10), cyanidin-3-*O*-(6-*O*-acetyl)-glucoside (m11), malvidin-3-*O*-glucoside (m12), petunidin-3-*O*-(6-*O*-acetyl)-glucoside (m13), malvidin-3-*O*-(6-*O*-acetyl)-glucoside (m14), petunidin-3-*O*-(6-*p*-coumaroyl)-glucoside (m15), malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside (m16), malvidin-3,5-*O*-diglucoside (m17), gallic acid (m18), citric acid (m19), *E*-resveratrol (m20), *E*-piceatannol (m21), catechin (m22), epicatechin (m23), coutaric acid (m24) caftaric acid (m25), fertaric acid (m26), piceid (m27), catechin gallate (m28), kampferol-3-*O*-glucoside (m29), pallidol (m30), *E*-ε-viniferin (m31), quercetin-3-*O*-glucoside (m32), quercetin-3-*O*-glucuronide (m33), myricetin-hexoside 1 (m34), myricetin-glucoside (m35), quercetin derivative (m36), procyanidin B1 (m37), procyanidin B2 (m38), procyanidin B3 (m39), procyanidin B4 (m40), kaempferol-3-*O*-rutinoside (m41), procyanidin gallate 1 (m42), procyanidin trimer 2 (m43), procyanidin gallate 1 (m44), procyanidin gallate 2 (m45) as in Billet *et al.* (2018) and Martins *et al.* (2021).

5. RNA extraction and transcription analyses by real-time qPCR

Total RNA was extracted from approximately 0.2 g of freshly ground samples following the protocol described by Reid *et al.* (2006). RNA was purified using the GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, Massachusetts, United States of America) followed by DNAse I treatment (Thermo Fisher Scientific, Massachusetts, United States of America). Using 1 µg of total RNA, cDNA was synthesised utilising the Xpert cDNA Synthesis Kit Mastermix (Grisp, Porto, Portugal). The purity and concentration of RNA were evaluated using NanoDrop.

The expression of several targeted genes in berries and exocarps at different developmental stages from control and polyol-treated vines was analysed by real-time qPCR. Real-time qPCR analyses were performed with Xpert Fast SYBR PCR Kit (Grisp, Porto, Portugal) using 1 µl of cDNA (diluted

1:10 in ultra-pure water) in a final reaction volume of 10 µl per well. Experiments were performed in biological triplicates using as reference genes *VviACT1* (actin) and *VviGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as these genes were demonstrated to be highly stable and ideal for qPCR data normalisation in grapevine (Reid *et al.*, 2006). Melting curves were performed at the end of each run to confirm the absence of unspecific or primer-dimer amplification. Gene-specific primers used in this work are listed in Supplementary Table 1. The relative expression data were normalised by the average expression of the reference genes (Pfaffl, 2001) and analysed using the CFX Manager Software 2.0 (Bio-Rad, California, United States of America).

6. Protein Extraction

Total protein extraction from grape berry and exocarp powder was performed as described by Conde *et al.* (2016) with some adjustments. Sample powder was mixed with extraction buffer in an approximately 1:1 powder:buffer ratio. Protein extraction buffer contained 50 mM Tris-HCl pH 8.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT) and 0.1 % (v/v) Triton X-100. The homogenates were centrifuged at 18,000 × *g* for 20 min, and the supernatants were collected. Supernatants were purified using an Amicon Ultracell-3K (Millipore, Massachusetts, United States of America) to remove contaminants, kept on ice and used for all enzymatic assays. Total protein concentrations were assessed using Bradford's method, which used bovine serum albumin as a standard (Bradford, 1976).

7. UFGT, PAL, mannitol dehydrogenase (MTD) and sorbitol dehydrogenase (SDH) enzyme activity assays

The enzymatic activity of UFGT was assessed as described by Lister *et al.* (1996) and adapted by Conde *et al.* (2016). The assay mixture contained 300 mM Tris-HCl buffer (pH 8.0), 1 mM UDP-glucose, 200 µL of protein extract and 5 mM of DTT in a final volume of 1 mL. The reaction was initiated by the addition of 1 mM quercetin as substrate, corresponding to a saturating concentration. The reaction mixture was incubated in the dark under gentle agitation for 30 min. Following incubation, dilutions were prepared using 100 µL of each assay mixture and 900 µL of Tris-HCl reaction buffer to evaluate the production of quercetin-3-glucoside at 350 nm (ε = 21,877 M^{-1} cm^{-1}).

The enzyme activity of PAL was determined as described by Conde *et al.* (2016). The assay mixture contained 0.2 mL of protein crude extract and 3.6 mM NaCl in 50 mM Tris-HCl (pH 8.9) of reaction buffer in a final volume of 1 mL. Reactions were initiated by adding 25 mM of L-phenylalanine. The reaction mixture was incubated at 41 °C in the dark under gentle shaking for 30 min, and the formation of trans-cinnamic acid was assessed at 290 nm (ε = 17,400M^{-1} cm^{-1}).

The enzyme activity of MTD and SDH was assessed as described by Conde *et al.* (2015). The assay mixtures contained 200 µL of protein extract, 300 mM Bis-Tris Propane (pH 9.0), 1 mM NAD^+, and 200 mM D-mannitol or 200 mM sorbitol for MTD and SDH enzyme activity, respectively. The reduction of NAD^+ to NADH was evaluated spectrophotometrically at 340 nm. D-mannitol oxidation and sorbitol oxidation were initiated by the addition of the polyol. The MTD and SDH enzymatic activities in the direction of fructose reduction were performed precisely like the mannitol oxidation assay but using 200 mM fructose to initiate the reaction and 1 mM NADH as a co-factor. The reaction mixture was incubated at 37 °C in the dark under gentle agitation.

8. Quantification of anthocyanins

The extraction of anthocyanins was carried out using 20 mg of lyophilised tissue from each treatment and 90 % methanol (v/v), as described in Conde *et al.* (2016). Suspensions were vortexed for 10 min and maintained overnight at 4 °C. The samples were centrifuged at 18,000 × *g* for 20 min, and the supernatants recovered. From each experimental condition, 200 µL of supernatant was mixed with 1.8 mL of 25 mM KCl (pH = 1.0). The absorbance of the samples was measured at 520 and 700 nm. Total anthocyanin concentration was assessed in relation to cyanidin-3-glucoside (C-3-G) equivalents, as expressed in the equation:

$$[\text{Total anthocyanins}](\text{mg L}^{-1}) = \frac{(A_{520} - A_{700}) \times MW \times DF \times 1000}{\varepsilon \times 1}$$

where MW is the molecular weight of C-3-G (449.2 g mol^{-1}), DF is the dilution factor, and ε corresponds to the molar extinction coefficient of C-3-G (26,900 M^{-1} cm^{-1}). The concentration was subsequently presented per mg of dry weight (DW) of used exocarp tissue.

9. Quantification of total flavonoids

Quantification of flavonoids was carried out as described by Matejić *et al.* (2013) with some modifications. Assay mixtures contained 20 µL of standard quercetin solutions or methanolic extracts from the experimental conditions, 30 % ethanol, 0.2 % (w/v) of aluminium chloride and 20 mM of potassium acetate. After incubation at room temperature for 40 min in the dark, the absorbance of the reaction mixtures was assessed at 415 nm. Total flavonoid concentration in methanolic extracts was quantified using a quercetin hydrate calibration curve (5–100 µg mL^{-1}) and expressed as quercetin equivalents per gram of DW.

10. Quantification of total phenolics

The concentration of total phenolics was quantified by the Folin–Ciocalteu method in exocarps from all experimental conditions. Total phenolics were extracted using a ratio of 1.5 mL of methanol absolute per 20 mg of lyophilised tissue from each experimental condition. The homogenates were vigorously agitated for 30 min and maintained overnight in the dark at 4 °C. Subsequently, homogenates were centrifuged at 18,000 × *g* for 20 min. 20 µL of each supernatant was added to 1.58 mL of deionised water and 100 µL of Folin reagent, vigorously agitated and incubated for 5 min in the dark before adding 300 µL of 2 M sodium carbonate.

After 2 h of incubation in the dark, the absorbance was evaluated at 765 nm. Total phenolic concentrations were quantified using gallic acid equivalents (GAE) per gram of DW.

11. Statistical Analysis

The results were statistically analysed using Student's t-test resorting to GraphPad Prism 9 software (GraphPad Software Inc., USA). The results are shown as mean ± standard deviation of the mean (SD), and statistical comparisons were calculated with a 95 % confidence interval. Parametric tests were employed since all data sets have Gaussian distributions. For each test result, a P-value indicates the significance of each tested sample. This significance is indicated in the figures with $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$(***) or $P < 0.0001$(****). All results were obtained from the analysis of three biological replicates.

RESULTS

1. Effect of foliar application of polyols on phenological parameters

As observed in Table 1, control and polyol-treated berries exhibited similar pH, AWC and °Brix during the *véraison* (EL-35) and mature stages (EL-38) of development. However, the ABW was higher in polyol-treated berries compared to control berries. Polyol-treated berries were approximately 40 % and 20 % heavier than control berries during *véraison* and mature stages, respectively.

The treatment did not promote any statistically significant change in net photosynthetic rate (A), stomatal conductance (gs), transpiration rate (E), intercellular CO2 concentration/ ambient CO2 ratio (Ci/Ca) and A/gs ratio (intrinsic water use efficiency) as described in Table 2.

2. Foliar application of polyols changes the concentration of ABA in grape berries and exocarps

The concentration of ABA in the *véraison* stage (EL-35) was 226.97 pmoles g^{-1} FW in control berries compared to 792.33 pmoles g^{-1} FW of polyol-treated berries, which corresponded approximately to a 3.5-fold increase in polyol-treated berries. Similarly, mature (EL-38) treated berries exhibited a 6-fold increased ABA content compared to control berries, as shown in Figure 1A. Contrarily, in the berry exocarp, the application of a polyol solution did not produce any statistically significant alteration in the ABA content (Figure 1B) despite a visible but non-statistically difference at *véraison*.

3. Foliar application of polyols modifies the phenylpropanoid, stilbenoid and flavonoid pathways

The concentration of anthocyanins was significantly higher in exocarps of EL-35 and EL-38 berries from polyol-treated

TABLE 1. Phenological parameters of control and polyols-treated grape berries—pH, average berry weight (ABW), average water content (AWC) and °Brix. EL-35 (*Véraison*) and EL-38 (Mature) are the stages of fruit maturation. Values are the mean of ± SD of three biological replicates. The asterisk indicates statistical significance (Student's t-test; *P ≤ 0.05).

		pH		ABW (g)		AWC (%)		°Brix	
EL-35	Control	2.77 ± 0.19	ns	1.26 ± 0.19	*	81.6 ± 0.2	ns	13.4 ± 0.4	ns
	Polyols	2.77 ± 0.17		1.76 ± 0.23		82 ± 0.3		12.6 ± 0.3	
EL-38	Control	3.76 ± 0.14	ns	1.57 ± 0.12	*	72.9 ± 0.3	ns	22.3 ± 0.2	ns
	Polyols	3.71 ± 0.17		1.87 ± 0.14		74 ± 0.3		21.9 ± 0.3	

TABLE 2. Physiological parameters of field-grown grapevines (polyol-treated and control). Net photosynthetic rate (A), stomatal conductance (gs), transpiration rate (E), intercellular CO_2 concentration/ambient CO_2 ratio (Ci/Ca) and A/gs ratio.

		Transpiration rate (E) ($mmol.m^{-2}.s^{-1}$)			Stomatal conductance (gs) ($mmol.m^{-2}.s^{-1}$)			Net photosynthetic rate ($\mu mol.m^{-2}.s^{-1}$)			Intrinsic water use efficiency (A/gs) ($\mu mol.mol^{-1}$)			Ratio of intercellular and atmospheric CO_2 concentration (*Ci/Ca*)		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
July 16th	Control	5.39	1.10	ns	188.26	48.81	ns	11.59	2.14	ns	62.96	7.36	ns	0.64	0.03	ns
	Polyols	5.71	1.38		205.75	52.82		11.71	2.26		58.45	9.04		0.66	0.04	
July 30th	Control	4.02	1.39	ns	146.46	65.52	ns	10.45	4.02	ns	75.25	12.33	ns	0.63	0.04	ns
	Polyols	4.02	1.49		170.97	89.76		11.17	4.65		71.50	13.79		0.65	0.04	

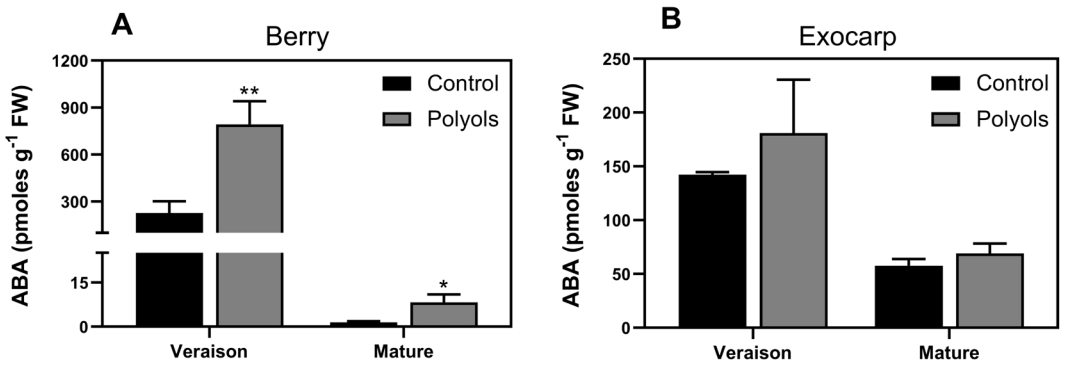


FIGURE 1. Foliar application of a polyol solution influences ABA concentration in grape berries (A) and exocarps (B). ABA accumulation in grape berries and exocarps in non-treated grapevine plants (Control) and grapevines sprayed with a 2 mM mannitol + 2 mM sorbitol solution (Polyols). *Véraison* (EL-35) and mature (EL-38) are the stages of fruit maturation. Values are the mean of ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05, **P ≤ 0.01).

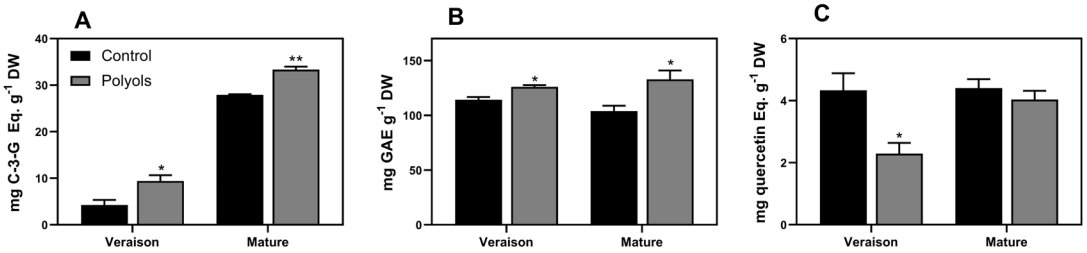


FIGURE 2. Foliar application of a polyol solution modifies the concentration of anthocyanins, phenolics and flavonoids in exocarps at *véraison* and mature stages. (A) Total anthocyanin concentration is presented as cyanidin-3-glucoside equivalents (C-3-G Eq.) per g of dry weight (DW). (B) Total phenolics concentration is presented as gallic acid equivalents (GAE Eq.) per g of DW. (C) Total flavonoid concentration is presented as quercetin equivalents per g of DW. Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance between berries of non-treated grapevine plants (Control) and berries from grapevines treated with a 2 mM mannitol + 2 mM sorbitol solution (Polyols). (Student's t-test; *P ≤ 0.05 and **P ≤ 0.01).

grapevines, containing 9.42 and 33.33 mg of cyanidin-3-glucoside equivalents per gram of DW, respectively, compared to control exocarps that presented only 4.23 and 27.9 mg of cyanidin-3-glucoside equivalents per gram of DW, respectively, as presented in the Figure 2A.

The concentration of phenolics was higher in the exocarp of berries harvested from polyol-treated grapevines, corresponding to 126.07 mg of gallic acid equivalents per gram of DW compared to 114.20 mg of gallic acid equivalents (GAE) per gram of DW of control exocarps in EL-35 (Figure 2B). Similarly, exocarps of EL-38 berries of polyols-treated grapevines exhibited a significantly higher concentration of phenolics compared to exocarps of berries of non-treated grapevines, displaying 132.75 mg GAE/g DW and 103.90 mg GAE/g DW, respectively. The concentration of flavonoids was not affected by foliar polyol application in exocarps of mature (EL-38) berries after an observed decrease in the EL-35 stage (Figure 2C).

The PAL enzyme activity (V_{max}) decreased by approximately 46 % in exocarps from polyol-treated grapevines compared to the exocarps of non-treated berries in the *véraison* (EL-35) stage. However, in the mature stage (EL-38), PAL enzyme activity in exocarps was approximately 3-fold higher in polyol-treated grapevines compared to the control (Figure 3A). An identical pattern was observed in the transcript abundance of *VviPAL1,* as shown in Figure 3B.

The concentration of stilbenes with one degree of polymerisation (DP1; sum of *E*-resveratrol, *E*-piceatannol and piceid) and two degrees of polymerisation (DP2; sum of pallidol and *E*-ε-viniferin), obtained by UPLC-MS, was significantly higher in mature exocarps of polyol-treated grapevines (Figure 4A,B), by ca. 2-fold in both cases. Similarly, the absolute quantification of piceid by HPLC revealed that its concentration in exocarps of mature berries from polyol-treated grapevines was more than 3-fold higher than the control, reaching almost 0.06 mg g^{-1} DW (Figure 4C). Moreover, the expression of *VviSTS1* increased 6-fold in exocarps (Figure 4D).

The relative concentration of total flavan-3-ols remained unchanged by polyol application (Figure 5A), as well

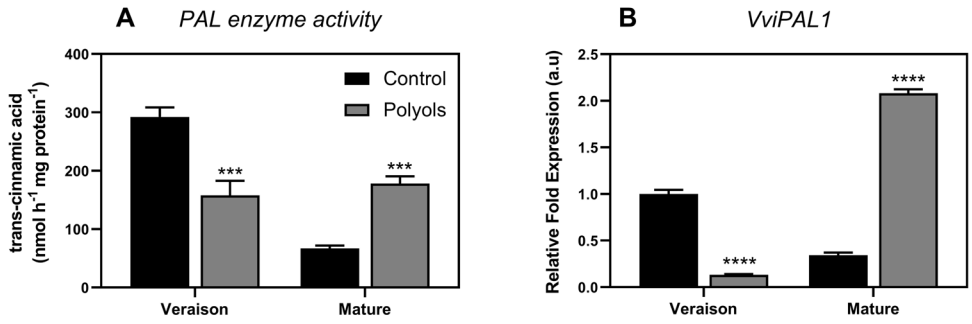


FIGURE 3. Foliar application of a polyol solution promotes changes in the phenylpropanoid pathway in exocarps at *véraison* and mature stages. (A) PAL enzyme activity was determined as V_{max} in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; ***P ≤ 0.001). (B) Transcript analysis of phenylalanine ammonia-lyase 1 (*VviPAL1*) in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Gene expression analyses were performed by real-time qPCR, and the results of *VviPAL1* relative expression were obtained upon normalisation with the expression of the reference genes *VviACT1* and *VviGAPDH*. Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; ****P ≤ 0.0001).

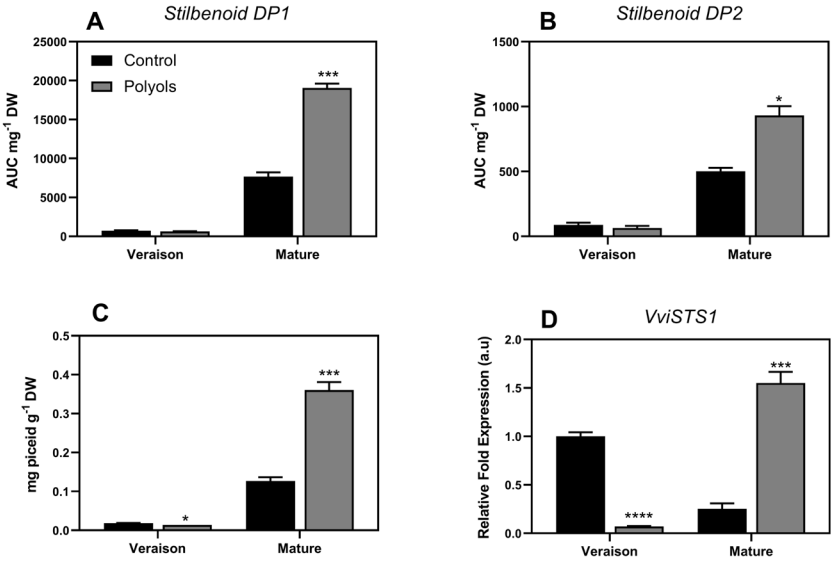


FIGURE 4. Foliar application of a polyol solution modifies stilbenoid pathway in exocarps at *véraison* and mature stages. The relative concentration of detected stilbenoids DP1 (*E*-resveratrol, *E*-piceatannol and piceid) (A) and stilbenoids DP2 (pallidol and E-e-viniferin) (B) in arbitrary units of concentration (AUC) per mg^{-1} of DW in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05, ***P ≤ 0.001). (C) HPLC analysis of the absolute concentration of piceid in mg of piceid per gram-1 of DW in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05, ***P ≤ 0.001). (D) Gene transcriptional analysis of grapevine stilbene synthase 1 (*VviSTS1*) in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). *VviSTS1* relative expression levels were obtained after normalisation with the expression of the reference genes *VviACT1* and *VviGAPDH*. Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; ***P ≤ 0.001, ****P ≤ 0.0001).

as the absolute concentration of catechin (Figure 5B). The expression of *VviLAR1* and *VviANR* decreased in mature exocarps from polyol-treated berries compared to non-treated ones (Figure 5C,E), while *VviLAR2* was downregulated at EL-35 but remained unaltered at El-38 (Figure 5D).

The absolute quantities of anthocyanins di-OH (cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, cyanidin-3-*O*-(6-*O*-acetyl)-glucoside) and tri-OH delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, petunidin-3-*O*-(6-*O*-acetyl)-glucoside, malvidin-3-*O*-(6-*O*-acetyl)-glucoside,

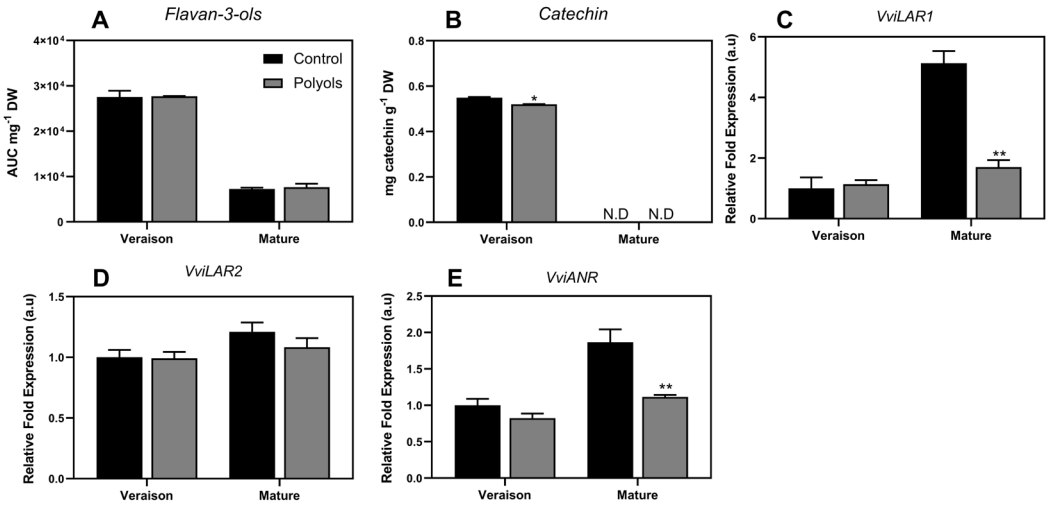


FIGURE 5. Foliar application of a polyol solution promotes changes in the flavonoid pathway in exocarps at *véraison* and mature stages. (A) UPLC-MS analysis of the relative concentration of flavan-3-ols (catechin, epicatechin, catechin gallate, procyanidin B1-4, procyanidin gallates and procyanidin trimers) in arbitrary units of concentration (AUC) per mg^{-1} of DW in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. (B) HPLC analysis of the absolute concentration of catechin in mg of catechin per gram-1 of DW in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05). Transcriptional analyses of grapevine leucoanthocyanidin reductase 1 (C) and 2 (D) (*VviLAR1* and *VviLAR2*, respectively) and the anthocyanidin reductase (E) (*VviANR*) in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). The relative expression levels of *VviLAR1*, *VviLAR2* and *VviANR* were obtained after normalisation with the expression of the reference genes *VviACT1* and *VviGAPDH*. Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; **P ≤ 0.01).

petunidin-3-*O*-(6-*p*-coumaroyl)-glucoside, malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside, malvidin-3,5-*O*-diglucoside) determined by UPLC-MS, increased in exocarps of polyol-treated berries compared to non-treated ones in both maturation stages (Figure 6A,B). The quantification of a specific anthocyanin, cyanidin-3-glucoside, by HPLC, showed an identical pattern (Figure 6C). Concordantly, the enzyme activity of UFGT in exocarps was much higher in polyol-treated grapevines when compared to the control, where it was not detected (Figure 6D). In the same way, the expression of *VviUFGT1* was clearly stimulated in exocarps from polyol-treated grapevines, demonstrated by a 2-fold increase in *véraison* and a 29 % increase in mature samples (Figure 6E**).**

4. Foliar application of polyols modifies mannitol and sorbitol metabolism

Polyol application in leaves increased mannitol oxidation rate by approximately 35 % via MTD and sorbitol oxidation via SDH 2-fold in mature berries compared to the control, as shown in Figure 7A,B**.** Contrarily, the fructose reduction rates of MTD and/or SDH decreased by 6-fold in polyol-treated berries, reaching a V_{max} of 0.004 µmol min^{-1} mg^{-1} protein in polyol-treated berries compared to non-treated berries (Figure 7C). Concordantly, the concentration of sorbitol in berries of polyols-treated grapevines clearly decreased in *véraison* and mature berries compared to the control, as shown in Figure 7D. The transcription analysis of *VviMTD1* showed a 50 % decrease in these transcripts in mature polyol-treated berries (Figure 7E).

5. Modifications of the quantities of secondary metabolites and amino acids induced by foliar application of polyols—an UPLC-MS-based metabolomics analysis

An extensive UPLC-MS-based metabolomics analysis on polyol-treated exocarps allowed the detection of 6 amino acids, 5 phenolic acids, 5 stilbenoids, 7 flavonols, 11 flavan-3-ols and 11 anthocyanins. Changes in relative quantities of these metabolites are demonstrated in the heatmaps depicted in Figure 8. In exocarps, minor modifications in the amino acid content were observed. The concentration of phenolic acids slightly increased in mature exocarps, particularly fertaric acid. The quantities of stilbenoids DP1 (*E*-resveratrol, *E*-piceatannol and piceid and DP2 (pallidol and *E*-ε-viniferin) were substantially increased in exocarps at EL-38 stage. Importantly, in the exocarps from polyol-treated berries a significant increase in the amount of many specific flavonols (e.g., myricetin-hexoside 1), flavan-3-ols (e.g., procyanidin B2 and B3), anthocyanins di-OH (cyanidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-(6-*O*-acetyl)-glucoside) and anthocyanins tri-OH (e.g., malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside) was observed either at EL-35 or EL-38 stages and at both stages in the case of several of these metabolites. The increased accumulation

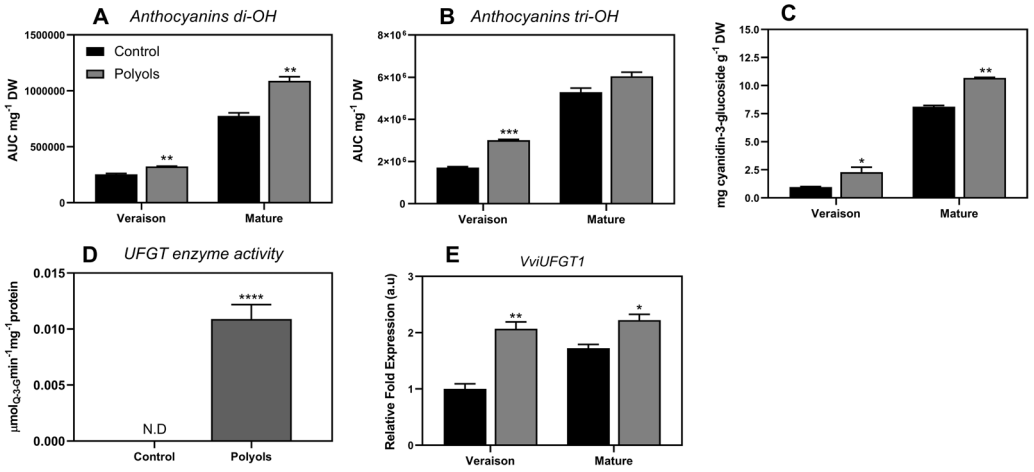


FIGURE 6. Foliar application of a polyol solution increases biosynthesis of anthocyanins in exocarps during *véraison* and mature stages. UPLC-MS analysis of the total concentration of anthocyanins di-OH (A) and anthocyanin tri-OH (B) in arbitrary units of concentration (AUC) per mg^{-1} of DW in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; **P ≤ 0.01, ***P ≤ 0.001). (C) HPLC analysis of the total concentration of cyanidin-3-glucoside in mg of cyanidin-3-glucoside per $gram^{-1}$ of dry weight in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05, **P ≤ 0.01). (D) UFGT total enzyme activity was determined as V_{max} in exocarps of mature berries from non-treated grapevines (Control) and treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; ****P ≤ 0.0001). (E) Transcript levels of UDP-glucose:flavonol 3-*O*-glucosyltransferase (*VviUFGT1*) in exocarps of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). *VviUFGT1* relative expression levels were obtained after normalisation with the expression of reference genes *VviACT1* and *VviGAPDH*. Values are presented as mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05, **P ≤ 0.01).

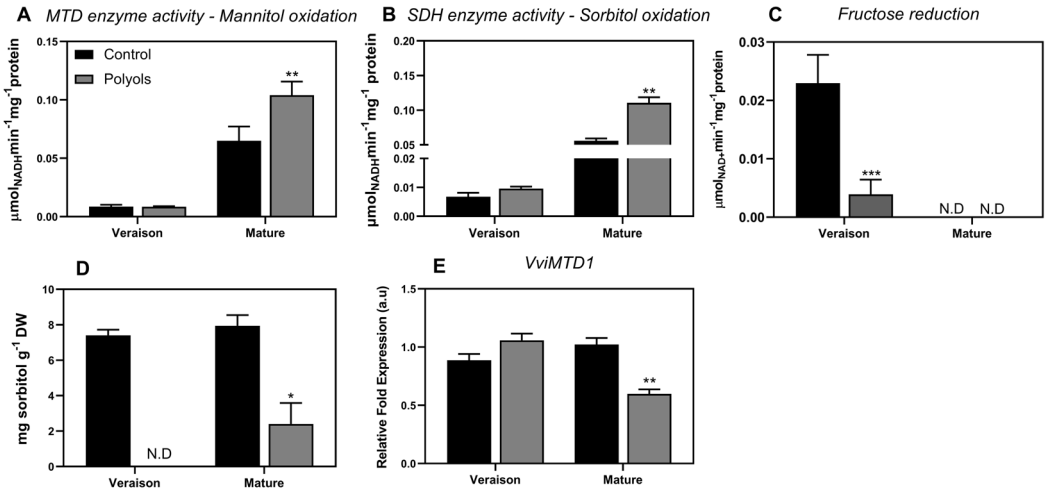


FIGURE 7. Foliar application of a polyol solution changes the biochemical activity of MTD and SDH in berries at *véraison* and mature stages. V_{max} of mannitol oxidation (A), V_{max} of sorbitol oxidation (B) and fructose reduction (C) in grape berries at véraison and mature stages of maturation. Values are presented as mean ± SD of three biological replicates. N.D—Non-detected. Asterisks indicate statistical significance (Student's t-test; **P ≤ 0.01, ***P ≤ 0.001). (D) HPLC analysis of the total concentration of sorbitol in mg of sorbitol per gram-1 of dry weight in berries of non-treated grapevines (Control) and polyol-treated grapevines (Polyols). Values are the mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; *P ≤ 0.05). (E) Transcript levels of *VviMTD1* of polyol-treated berries (Polyols) and non-treated berries (Control). *VviMTD1* relative expression was obtained after normalisation with the expression of the reference genes *VviACT1* and *VviGAPDH*. Values are presented as mean ± SD of three biological replicates. Asterisks indicate statistical significance (Student's t-test; **P ≤ 0.01).

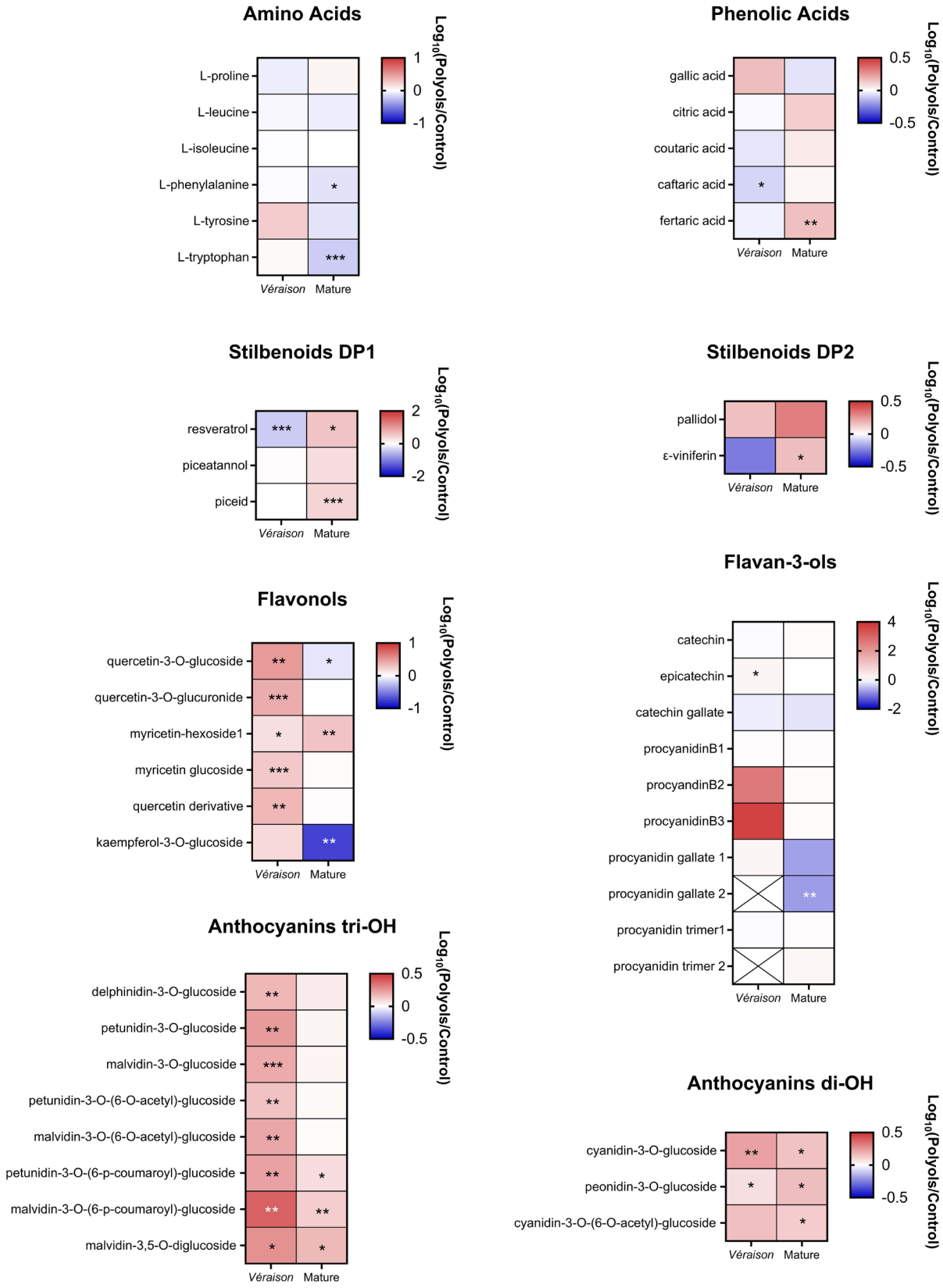


FIGURE 8. Foliar application of a polyol solution modifies the quantities of amino acids and secondary metabolites in *véraison* and mature exocarps. Metabolomics analysis was performed by UPLC-MS. The values of the metabolites of exocarps at *véraison* and mature stages of development are presented as Log10(Polyols/Control). The different colours represent an increase (red) or a decrease (blue) of the different metabolites indicated. Three biological replicates were used, and asterisks indicate statistical significance (Student's t-test; *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001) in each tissue and each developmental stage.

of these specifically identified secondary metabolites agrees with the quantification of total phenolics, total stilbenoids DP1 and DP2, and total anthocyanins di-OH and tri-OH described above.

DISCUSSION

1. Foliar application of polyols increases ABA concentration in berries and exocarps

Ongoing climate changes demand the development of different sustainable viticulture strategies to improve grapevine adaptability while maintaining or improving the yield and quality of the production (Bernardo *et al.*, 2018). The physiological role and mechanisms of polyols, namely mannitol and sorbitol, as a physiological strategy for water stress tolerance in grapevine were described by Conde *et al.* (2015), however, the potential of the exogenous application of polyols as an abiotic stress mitigation strategy or as biostimulants improving berry quality was so far unknown. To address this hypothesis, we studied the effects of a polyol solution applied at a foliar level containing low concentrations of mannitol and sorbitol (2 mM).

ABA hormone is an important regulator of several stress/adaptative-specific genes and, consequently, responsible for the upregulation of important molecular mechanisms that play an important role in plant survival under different environmental conditions, including water-deficit stress environments (Cochetel *et al.*, 2020; Ferrandino & Lovisolo, 2014). The observed upregulation of ABA biosynthesis in grape berries following foliar application of exogenous polyols is a new finding likely to have important agronomical implications. From the scientific standpoint, it is tempting to speculate that exogenous mannitol and/or sorbitol is sensed and triggers ABA synthesis, one of the grapevine's first responses to water deficit; however, this cause–effect relationship deserves further attention in future studies. ABA upregulation triggered by polyol application might also have been pivotal for the upregulation of several berry quality-associated secondary metabolic pathways that were also observed in this study, as discussed below.

2. Foliar application of polyols stimulates phenylpropanoid, stilbenoid and anthocyanin synthetic pathways in grape exocarps

Polyols foliar application enhanced the responsiveness of the mechanisms involved in the phenylpropanoid pathway in the exocarps through the elicitation/activation of key intervenient enzymes, consequently allowing greater availability of precursor molecules for the biosynthesis of stilbenes and anthocyanins. This was the case of PAL, which catalyses the first step of the phenylpropanoid pathway, as denoted by a strong upregulation of *VvPAL1* occurring in mature polyol-treated berry skins together with a significant increase of the biochemical activity of PAL measured in protein extracts and of numerous polyphenols quantified by UPLC-MS, particularly stilbenoids and anthocyanins.

The regulation of stilbenoid biosynthesis is modulated by biotic and abiotic factors (Rienth *et al.*, 2021; Savoi *et al.*, 2016). In this work, the foliar application of polyols stimulated the production of stilbene compounds in exocarps, especially at the mature stage (EL-38). In particular, an increased concentration of total stilbenoid DP1 and DP2 in treated exocarps was observed, in accordance with the significant increase of *VviSTS1* transcripts. These results agree with the apparent increase of precursor phenylpropanoids synthesised in the phenylpropanoid pathway presented in Figure 3 but also with the increased content of ABA presented in Figure 1 (Miliordos *et al.*, 2022). These data suggest that the application of polyols constitutes a strategy to produce an increased concentration of stilbenes in exocarps. The increased concentration of stilbenoid compounds in exocarps constitutes an important attribute, considering these molecules are particularly active in biotic stress responses by enhancing tolerance against grapevine powdery mildew, for instance. In this regard, these results pave the way for new studies to evaluate the protective effect of exogenous polyols against vine pathogen-induced diseases. Moreover, the nutraceutical and pharmacological properties of stilbenes are recognised and described in several studies (Reinisalo *et al.*, 2015; Sirerol *et al.*, 2016), reinforcing the importance of the present findings.

ABA has been described as an activator of anthocyanin biosynthesis genes and anthocyanin-related transcription factors (Badim *et al.*, 2022; Gagné *et al.*, 2011; Koyama *et al.*, 2018; Sun *et al.*, 2019), so it is possible that the observed changes in anthocyanin levels were mediated by ABA signalling upon sensing of exogenously applied polyols in leaves. The observed increased expression of *VviUFGT1* paralleled with the enhanced enzyme activity of UFGT in exocarps, where the biosynthesis of anthocyanins predominantly occurs (Gouot *et al.*, 2019; Walker *et al.*, 2006). The expression of genes such as F'3'H, F'35'H, LDOX, DFR and UFGT, involved in the flavonoid pathway and anthocyanin biosynthesis, are normally upregulated in water-deficit, thus, ABA-triggering conditions (Castellarin *et al.*, 2007a; Castellarin *et al.*, 2007b; Deluc *et al.*, 2009; Ferrandino and Lovisolo, 2014). Interestingly, while the quantities of flavan-3-ols were more than 3-fold lower in mature exocarps than in *véraison* ones (Figure 5), with non-detected levels in the specific case of catechin, the transcripts of *VviLAR2* were similar, and those of *VviANR* and *VviLAR1* were even higher, despite their downregulation by polyol application. This observation suggests that the synthesis of these compounds is strongly regulated at a post-transcriptional level.

3. Foliar application of polyols enhances polyol intracellular oxidation into energy-yielding, hence secondary metabolite biosynthesis

The targeted metabolomics analysis performed in our previous study (Conde *et al.*, 2015) showed that Tempranillo cv. grapevines under drought stress accumulate a higher-than-normal amount of polyols in the grape berry, including

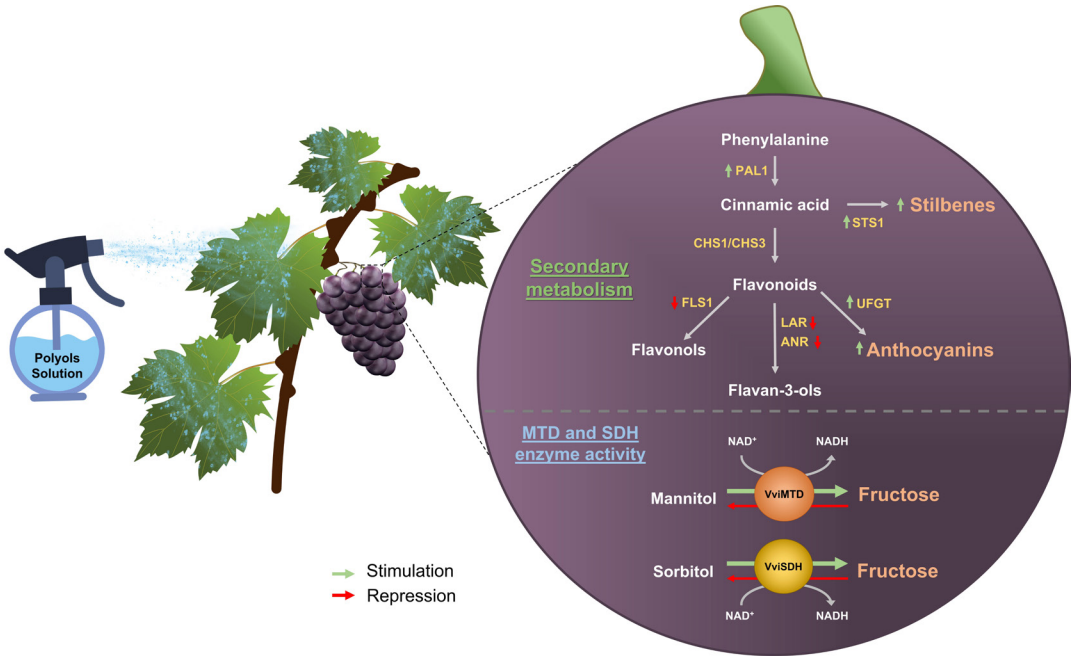


FIGURE 9. The foliar application of a polyol solution in grapevines stimulates the synthesis of stilbenes and anthocyanins and modulates MTD and SDH enzyme activities. The biosynthesis of stilbenes (STS) and anthocyanins (UFGT) is positively regulated by the foliar application of a polyol solution containing mannitol and sorbitol. Moreover, the application of polyols stimulates the synthesis of fructose (via mannitol or sorbitol oxidation) by MTD and SDH actions in grape berries.

mannitol, sorbitol, galactinol and dulcitol. At the molecular level, the accumulation of polyols was found to be mediated by the stimulation of mannitol transport at the plasma membrane via VviPLT1 (VviPMT5) coordinated with repression of the intracellular oxidation of mannitol/sorbitol into fructose, mediated by mannitol/sorbitol dehydrogenases, while the reverse reaction of the enzymes (reduction of fructose) was stimulated. Thus, in stressed vines, the total amount of polyols accumulated in mature berries was approximately equivalent to a normal sucrose concentration in a mature berry, but this value could be even higher because many other (not quantified) polyols could account for this intracellular polyol pool under drought stress. Thus, in the present study, the observed activation of polyol intracellular oxidation (up-regulation of mannitol and sorbitol dehydrogenases; strong decrease in sorbitol content) in berries from mannitol-treated Touriga Nacional cv. plants follow an opposite trend and deserve further attention, particularly into the possibility of a protective role of polyols that may be, in fact, cultivar-dependent. It is also tempting to speculate that exogenous mannitol and sorbitol drive the metabolism of the intracellular pool of polyols to energy-yielding reactions or to fuel the biosynthesis of secondary metabolites, including stilbenoids and anthocyanins. Supporting this theory appears to be the fact that berries from polyol-treated plants were approximately 40 % heavier than control ones despite the absence of differences in their water content percentages.

4. Is mannitol a biostimulant of grapevine?

The mechanisms underlying the apparent biostimulant effect of exogenous mannitol and sorbitol still need further research, but they share some similarities with the ones reported before for exogenously applied kaolin (Conde *et al.*, 2016). The application of this partially light-reflecting clay induced a set of molecular and biochemical changes that together represented a significant stimulation of phenylpropanoid and flavonoid pathways in grapevine, including an increase in the concentration of anthocyanins in mature berries of plants that were also more protected against water deficit situations (Conde *et al.*, 2016). Seaweed (*Ascophyllum nodosum*) extracts are also biostimulants of grapevine secondary metabolism and berry quality, making plants that were treated at the foliar level more able to cope with abiotic and biotic stresses (Frioni *et al.*, 2019; Salvi *et al.*, 2019). The berries from these plants (Sangiovese cv.) presented higher phenolic and anthocyanin concentrations at harvest, with several molecular mechanisms underlying the synthesis of these compounds upregulated. Chitosan application in grapevines also demonstrated similar effects in phenolic and anthocyanin concentrations and respective biosynthetic pathways, being, therefore, another example of a compound with biostimulant properties in this plant species (Singh *et al.*, 2020). Considering the results, besides having a biostimulant effect, exogenous application of polyols might even be an interesting strategy to improve freezing tolerance in grapes, as it led to a significant increase in ABA concentration.

This phytohormone has a pivotal role in bud dormancy and suffers an abrupt decrease in its concentration during bud break, which increasingly occurs earlier in the vegetative year as sudden climatic changes from freezing to warm conditions are more common (Zheng *et al.*, 2015).

Mannitol has a recognised role in plant–pathogen interaction, and its concentration increases significantly during plant infection by biotrophic or necrotrophic fungi, together with an increased expression of genes involved in the mannitol–synthetic pathway (Calmes *et al.*, 2013; Chaturvedi *et al.*, 1996; Dulermo *et al.*, 2009; Jobic *et al.*, 2007; Voegele *et al.*, 2005). For instance, stilbenes are key defensive metabolites involved in the resistance of grapevine to major fungal pathogens that include *Botrytis cinerea* (grey mould), *Plasmopara viticola* (downy mildew) and *Erysiphe necator* (powdery mildew). Grapevine varieties that are more resistant to biotic stress respond very rapidly to infection by producing high concentrations of stilbenes, including δ-viniferin and pterostilbene, at the sites of infection (Viret *et al.*, 2018). Thus, exogenous mannitol may mimic the presence of mannitol-producing plant pathogens, triggering and eliciting a response that involves the synthesis of stilbenoids and other biotic stress-protecting secondary metabolites. Reinforcing this hypothesis, stilbene synthesis, including piceid concentration and *VvSTS1* expression, was highly increased in berry exocarps from polyol-treated plants, displaying some of the most significant responses observed in this study.

CONCLUSION

In conclusion, the foliar application of a polyol solution containing 2 mM of sorbitol and 2 mM of mannitol stimulated ABA synthesis in grape berries, and the concentration of anthocyanins, stilbenes and total phenolics in exocarps, through the upregulation of phenylpropanoids and particularly the stilbenoid and anthocyanin pathways. Thus, exogenously applied polyols acted as biostimulants of important pathways of the secondary metabolism that enhanced key grape berry quality traits and potential ability to cope with stress. As neither control nor polyol-treated plants were in water deficit conditions, this stimulatory effect of polyols in pathways of the secondary metabolism is probably, at least in this case, not by a direct protective effect against abiotic stress, but due to a sort of "immunisation effect" obtained by plants sensing of low concentration mannitol and sorbitol that elicited a set of responses that made them more adapted to surrounding edaphoclimatic conditions. These findings indicate that foliar application of low-concentration polyols is a promising strategy to improve grape berry quality with the additional advantage of being safe for human health and the environment and relatively cost-efficient. It also represents a potential strategy to improve agronomical traits in other plant species.

However, it is important to point out that these results were obtained from experiments performed in just one season. Therefore, while the biostimulant effect of exogenously applied polyols is strongly suggested by the combination and integration of all reported results, repeating these experiments in additional seasons is important to solidify this conclusion further. In that regard, these efforts are currently ongoing, and these approaches, with an emphasis on UPLC-MS secondary metabolome profiling, will be repeated.

AUTHOR CONTRIBUTIONS

AC conceptualised the work, performed the experiments, and wrote and reviewed the manuscript. HB performed the experiments and wrote the manuscript. LTD, JMP, MF and MU performed the experiments. AL performed the experiments and reviewed the manuscript. HG conceptualised the work and wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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